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Cryptic translocation identification in human and mouse using several telomeric multiplex FISH (TM-FISH) strategies.

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Running title: Telomeric multiplex FISH (TM-FISH)

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Abstract.

Experimental data published in recent years showed that up to 10% of all cases with mild to severe idiopathic mental retardation may result from small rearrangements of the subtelomeric regions of human chromosomes. To detect such cryptic translocations, we developed a "telomeric" multiplex FISH assay, using a set of previously published and commercially available subtelomeric probes. This set of probes includes 41 cosmid/PAC/P1 clones located from less than 100kb to about 1 Mb from the end of the chromosomes. Similarly, a published mouse probe set, comprised of BACs hybridizing to the closest known marker toward the centromere and telomere of each mouse chromosome, was used to develop a mouse-specific "telomeric" M-FISH. Three different combinatorial labeling strategies were used to simultaneously detect all human sub-telomeric regions on one slide. The simplest approach uses only three fluors, and can be performed in laboratories lacking sophisticated imaging equipment or personnel highly trained in cytogenetics. A standard fluorescence microscope equipped with only three filters is sufficient. Fluor-dUTPs and labeled probes can be custom-made, thus dramatically reducing costs. Images can be prepared using generic imaging software (Adobe Photoshop), and analysis performed by simple visual inspection.

Abbreviations:
BAC = bacterial artificial chromosome
BIO = biotin
DEAC = diethyl aminomethyl coumarin
DIG = digoxigenin
DNP = dinitrophenyl
DOP-PCR = degenerate oligonucleotide priming PCR
DTAF = dichlorotriazinylfluorescein
FISH = fluorescence in situ hybridization
FITC = fluorescein isothiocyanate
M-FISH = multiplex FISH
TM-FISH = telomeric multiplex FISH
mTM-FISH = mouse TM-FISH
R6G = carboxyrhodamine6G

Keywords: telomere, M-FISH, fluorescence, cryptic translocation, mouse chromosomes, human chromosomes

Introduction

It is estimated that 7.4% of all cases with mild to severe idiopathic mental retardation result from small rearrangements of the subtelomeric regions of human chromosomes (Flint et al, 1995; Knight et al, 1999; Slavotinek et al, 1999). Rearrangements involving fewer than than 1-2 megabases are usually undetectable by cytogenetic banding techniques or molecular painting methods such as M-FISH and spectral karyotyping (Azofeifa et al, 2000; Schrock et al, 1996; Speicher et al, 1996). Such small translocations or deletions are easier and more conveniently detected by FISH, using subtelomeric chromosome-specific probes (Bacino et al, 2000; Ballif et al, 2000; Ghaffari et al, 1998; Granzow et al, 2000; Knight et al, 1997). Using a set of previously published probes, we developed three different telomeric multiplex FISH (TM-FISH) strategies that allow simultaneous hybridization of all 41 sub-telomeric probes on one slide. These commercially available probes (ATCC) include previously published cosmids, P1 and PAC clones (Anonymous, 1996). All these probes are known to be located at distances between 100 kb and 1 Mb from the end of the chromosomes. We also developed a similar M-FISH approach for mouse chromosomes, using a set of BAC probes (Korenberg et al, 1999) located at the centromeric and distal telomeric ends of the telocentric mouse chromosome. These probes correspond to the most telomeric and centromeric markers known on mouse chromosomes. We refer to this as mouse TM-FISH (mTM-FISH) to differentiate it from a true TM-FISH approach, as described for the human chromosomes. Although the physical distance between each probe in the set and its corresponding chromosome end is not yet known, this mouse set can be used for mapping purposes or in screening for chromosomal aberrations in murine embryonic stem cells (Henegariu et al, unpublished observations). Because TM-FISH uses commercially available probes (ATCC, Research Genetics), they can be prepared and labeled in any laboratory at a cost per analysis significantly less than regular M-FISH. The various fluor-dUTPs used for probe labeling can also be custom-prepared in any laboratory at a very reduced cost (Henegariu et al, 2000). TM-FISH can be performed without the need for specialized hardware or software and with increased sensitivity of detection of small translocations compared to regular M-FISH.

Results and discussion.

Simultaneous use of 40 (mouse) or 41 (human) unique probes in FISH raised problems not seen with chromosome painting probes. The human set included clones which yielded weaker fluorescent signals (especially for chromosomes 5, 10p, 11, 16, 19,

20, XY). Most of these were cosmids, which carry smaller inserts than a BAC or PAC. Two labeling procedures were tested and compared, nick translation and degenerate oligonucleotide priming PCR (DOP-PCR), each with distinct advantages and disadvantages. In nick translation, all probes tagged by the same haptene can be simultaneously labeled in the same vial, thus reducing the procedure to only five separate reactions. The main drawback is that nick translation requires periodical probe DNA isolation. DOP-PCR has the advantage of providing a virtually unlimited source of DNA template, which can be re-amplified when needed. The main disadvantage is that only a limited number of probes can be reproducibly labeled by DOP-PCR in the same vial, thus . labeling requires several separate reactions with each fluorophore. If template DNA from too many probes (10-15) is added in the same vial, PCR-labeling is poor, with many hybridization signals missing. DOP-PCR amplification and labeling of the human set of probes resulted in consistent FISH signals mostly when probes were labeled individually. Otherwise results were relatively poor, especially for the cosmids. Nick translation yielded more consistent hybridization signals and was chosen as labeling method for TM-FISH. Even after nick translation some cosmid probes still yielded weaker hybridization signals during TM-FISH. In order to adjust all signals to comparable intensities, two to five times higher amounts of labeled DNA from those probes was necessary during hybridizations, compared with P1 or PAC DNA. In the case of mTM-FISH, as the BACs have an average size of about 150 kb, DOP-PCR on sets of 4-5 probes/vial could be successfully used as labeling procedure (Fig 2d-f) and yielded robust hybridization signal. Recent publication of a new human telomere set including BACs and PACs (Knight et al, 2000) should make it possible to apply DOP-PCR labeling for TM-FISH as well.

Whereas the mouse probes were used in a five color assay, for the human probes we developed three different strategies of analysis (Table 1), each with advantages and disadvantages. Laboratories can choose one strategy over the other based on their experience with FISH procedures, training of their personnel in chromosome identification and quality of equipment.

One set/five fluors approach ("1/5"). In this true M-FISH-like procedure (Table 1), the human or mouse probe sets were labeled using two fluorophores, fluorescein isothiocyanate (FITC) and carboxyrhodamine6G (R6G), and three non-fluorescent haptenes, which required fluorescent antibody detection. The haptenes used were: dinitrophenyl (DNP, detected with anti-DNP-Cy3.5), biotin (BIO, detected with avidin-Cy5) and digoxigenin (DIG, detected with anti-DIG-diethyl aminomethyl coumarin [DEAC]). The combinatorial labeling scheme used (detailed in Fig. 1) results in a 23 color FISH assay, in which the p and q probes of the same chromosome are detected with

the same color combination. As some of the probes yielded very weak signals when in multiple combinations, the assay was made more robust by detecting not only the haptenes but also FITC and R6G with fluorescence-labeled antibodies, thus resembling a five-haptene system. In other words, primary antibodies against fluorescein and rhodamine were detected with secondary antibodies labeled with fluors of similar wavelength (dichlorotriazinylfluorescein [DTAF] for FITC and Cy3 for R6G). Regular M-FISH and TM-FISH were performed on a control case carrying a very small unbalanced translocation (Fig 1a-b). The telomeric assay positively identified the translocation in 92% of all metaphases examined, whereas regular M-FISH analysis identified the origin of the translocated fragment in less than 60% of metaphases. The reason for this difference is that the painting probes may not be able to detect translocated chromosomal fragments smaller than 2-3 Mb (Azofeifa et al, 2000). Thus, FISH with subtelomeric probes appears to be the method of choice for in situ cryptic translocation detection. M-FISH and TM-FISH performed on a set of fourteen cytogenetically normal samples from patients with autism did not identify any translocation. More patients are currently under analysis.

The mouse-specific telomeric assay was tested on a cytogenetic sample carrying a translocation. Cells in methanol:acetic acid from three mouse samples (two normal and one with translocation) were sent to our laboratory for a blinded study, without disclosing the name of the chromosomes involved. mTM-FISH required only one hybridization on each slide to identify the normal and abnormal samples and the chromosomes involved in the translocation (Fig. 2d-f), thus confirming the results obtained previously by G banding.

Two sets/three fluors approach ("2/3"). This technique uses the same probe combinations as in "1/5" (Fig. 1 and Table 1, columns F,R,N,B and D), but the probes are divided into two groups which are hybridized onto two different 22x22 mm areas of the same slide. The first group includes a probe set labeled with BIO and DIG (Table 1, columns F,R), whereas the second group consists of a different probe set, labeled with the three haptenes, BIO, DIG and DNP (Table 1, columns N,B and D). The reason for replacing the two fluors with haptenes in the first group is to decrease the total number of antibodies used for detection. This is only possible because the two groups of probes are hybridized separately. Thus, antibody detection on both areas of the slide is performed simultaneously using the same mixture of three fluorescence-labeled antibodies, against BIO, DIG and DNP. It is important to notice that, because of the labeling algorithm (Table 1), neither the first, nor the second group of probes set can identify the chromosomes by itself, only their combination. For example, the probes for chromosomes 2 and 11 are both labeled with BIO and DIG in the first group. After

hybridization, the color of the subtelomeric regions of chromosomes 2 and 11 will be identical, so this hybridization itself will not detect a t(2;11). However, in the hybridization on the second area of the slide (using the second group of probes), only the probes for chromosome 2 are again labeled (with DNP). These probes will have the same color as chromosome 20 probes (also labeled with DNP in the second group). However, chromosome 20 probes are not labeled in the first group, and thus chromosomes 2, 11 and 20 can be separated. In conclusion, the combined information from BOTH hybridizations identifies all telomeres. The hybridization signals of the three channels captured from one area, and two channels captured from the other area are merged into two separate color images using Adobe Photoshop, and the telomeres identified by comparisons with the algorithm in the table. Because it uses only three labeled antibodies (and thus only three colors) to detect the haptenes, this procedure is simpler and more robust than "1/5". The other advantage is that it can be performed on any fluorescence microscope equipped with only three filters.

Three sets/three fluors approach ("3/3"). This approach was designed with the purpose of providing an assay which does not require sophisticated equipment or personnel trained in chromosome identification. The probes are divided into three sets (Sets #1, #2 and #3, Table 1 and Fig. 2a-c), each detecting seven or eight chromosomes *independently* from the other two sets. Probes are labeled with the same three haptenes (BIO, DIG, DNP), and the sets are hybridized on separate areas of the *same* slide. Combinatorial labeling with three colors yields a maximum of seven combinations, thus allowing simple visual identification of every chromosome, even when a person has no prior training in differentiating human chromosome based on their DAPI staining. However, based solely on combinatorial labeling, theoretically, only 21 chromosomes can be detected in three independent sets. Because the human complement includes 23 pairs of chromosomes, the remaining two chromosome pairs were detected by ratio labeling (or signal strength), one of them in each of the sets #1 and #2. This was possible, because the telomeric probes of chromosomes 20 (set #1) and 19 (set #2) yielded much weaker signals than probes for chromosomes 4 (set #1) and 7 (set #2).

Imaging limitations and color display. For the five fluor procedure, 1/5, a microscope equipped with 5 different fluorescence filters and a CCD camera is necessary, whereas for procedures 2/3 and 3/3, any fluorescence microscope equipped with the common three filters is sufficient. In this case, a red, a green and a blue fluor (AMCA) can be chosen to detect the haptenes, whereas DAPI counterstaining is performed *after* imaging the FISH signals. Availability of a microscope with four filters, allows a choice of three fluors in the visible spectrum *and* DAPI staining. This scenario makes possible the use of

a simple digital camera for image capturing (Henegariu et al, 1999). Although specialized software for chromosome classification based on telomeric signals would be useful, figures 1 and 2 show that generic imaging software (Photoshop) is sufficient. However, merging and pseudocoloring the grayscale images of all five fluors plus DAPI (as in the 1/5 approach) in Photoshop yielded FISH signals with colors difficult or impossible to differentiate among some of the chromosomes. Simply merging five or six channels in a multicolor Photoshop image does not seem to be a robust enough approach for discriminating the colors of telomere signals, although it was sufficient for chromosome painting probes (Henegariu et al, 1999). The main reason is that the signals from the telomere probes are dot-like signals, unequal in size and intensity, which vary significantly even from one metaphase to the other. Therefore, a true 24 color image would require specialized software, allowing localized signal enhancement and proportional pseudocoloring. To allow analysis while still using Photoshop, we grouped and pseudocolored the grayscale images of the 1/5 analysis into two triplets: FITC, R6G and DAPI in a first color image, and DEAC, Cy3.5 and Cy5 in a second (Fig. 1 and 2). Merging only three channels (RGB image) allows the hybridization signals to be discriminated by color after simple visual inspection on the computer screen. By comparing the colors of the telomeres in the two images with the provided color chart, all chromosomes (and potential translocations) can be identified.

Materials and methods.

DNA probes and biological samples.

Probes used to detect the human subtelomeric regions are listed in Table 1, and were previously characterized (Anonymous, 1996). These probes are located from less than 100 to less than 1000 kb from the telomeres of their respective chromosomes. All probes are available from ATCC and Research Genetics. The mouse BACs listed in Table 1 were selected using genetic markers which define the centromeric and distal telomeric ends of the Whitehead/MIT recombinational maps of mouse chromosomes (Korenberg et al, 1999). As opposed to the human clones, the precise physical distance of the mouse BAC to the ends of the chromosomes is not yet known. The labeling combinations used for both mouse and human probes are detailed in Table 1.

The various human TM-FISH procedures were performed on several normal control samples, on fourteen samples from patients with autism (normal karyotypes) and on a known translocation sample. The latter was a patient carrying a small translocation on the tip of 1q [46,XX add(1)(1q41)], identified cytogenetically. G-banding could not detect the origin of the small translocated fragment. M-FISH and TM-FISH were separately used to identify the origin of the translocated fragment. Mouse TM-FISH was performed on two normal cytogenetic samples and on splenocytes from a 12Gso mouse, known to carry the (4;9) (B3;D) translocation. At the time of the analysis, we did not know which sample contained the translocation or which were the chromosomes involved. mTM-FISH correctly identified the translocation.

Slide and probe preparation, and labeling strategies.

Slides were prepared according to common cytogenetic procedures, with several modifications aimed at yielding very "flat" nuclei and chromosomes detailed elsewhere (Henegariu et al, 2001). Briefly, the slide was kept a few seconds in the hot water vapors of a waterbath at 75 C, then a few drops of cell suspension in fixative (3:1 methanol:acetic acid) were pipetted on the slide. As soon as the fixative started to dry and the cells on the glass surface became visible ("grainy" aspect), 4-5 drops of acetic acid were quickly placed on the slide, allowed to spread, and the slide was exposed again for 3-4 seconds to the hot water vapors. Then, the slide was quickly dried on a hot metal surface (65-70 C). The flatness of the cytogenetic preparation was important, as it allowed imaging of all hybridized probes in the same focal plane. Probes were labeled by nick translation or DOP-PCR (Telenius et al, 1992) using dUTP labeled with FITC, R6G, BIO, DIG and DNP. The labeled dUTP were custom synthesized in our laboratory by chemical conjugation reactions between reactive allylamine dUTP and succinimidyl ester of fluor/hapetene derivatives (Henegariu et al, 2000). 20-100 ng labeled probe DNA were

used for FISH, regardless of the method of labeling. Probes yielding weak signals, such as those for human chromosomes 10p, 19, X and Y were used in quantities 2-5 times higher than other probes. Probe DNA was prepared by alkaline lysis, using midi/maxiprep kits (Qiagen, Clontech). The following strategies were used to prepare the probe cocktails for TM-FISH.

Nick translation labeling: To initially test the quality of the various human probes, equal amounts of probe DNA (50-100ng each) for the p and q arms of the first twelve chromosomes were pooled together and labeled into two separate nick translation reactions. The p probes were labeled with BIO and the q probes with DIG, then mixed together, precipitated and hybridized on a normal control slide. After hybridization and antibody detection, the chromosomes were examined for the presence or absence of signals. Particular notice was taken of the probes yielding weak signals (the strength of a signal did not need precise quantification, only visual approximation). The same strategy was used to test the probes for the remaining chromosomes. Once this information was gathered, *probe cocktails* were prepared. As an example, for human 1/5 strategy, all probes to be labeled with a given haptene-or fluor-dUTP (Table 1) were pooled together, for a total of five probe cocktails. In every such pool, the probes yielding good signals were added at about 20-50 ng/hybridization, whereas 2-5 times more DNA was necessary for the probes found previously to yield weak signals. The total amount of DNA to be labeled was multiplied with the number of slides desired. In nick translation, there was no limit in reaction volume or amount of probe simultaneously labeled. The reaction worked equally well in a 15 or 50 ml tube incubated at 15 C in a waterbath. For any one hybridization, aliquots of DNA from each of the five labeled pools were mixed together, ethanol precipitated in the presence of 40-50 μg Cot-1 DNA (GIBCO), resuspended in hybridization buffer and hybridized overnight under a 22x22mm coverslip. After antibody detection and imaging, all signals were carefully accounted for. If, for example, the signal of a FITC labeled probe was weak or missing, a separate nick translation reaction using FITC-dUTP was performed for that probe, tested and mixed with the initial FITC-labeled pool. This strategy allowed convenient "repairing" of every probe cocktail made by nick translation.

DOP-PCR labeling. This technique was applied to the mouse clones. Using a known degenerate primer (Telenius et al, 1992), initial PCR reactions at low stringency were separately performed on every probe DNA. The PCR cycling included annealing temperatures of 15 C in the first cycle, 30 C in the next four cycles and 54 C for the remaining 25 cycles (Henegariu et al, 1999). For every BAC used, this DOP-PCR amplification provided the "PCR template" for the subsequent PCR labeling reactions. In our hands, mixing in the same tube the PCR templates of more than 4-5 probes for PCR labeling, resulted in a decrease of the hybridization signals. To prevent this, separate PCR

reactions with any labeled-dUTP were performed on one or maximum two probes per vial. Although this approach required dozens of labeling reactions, it preserved probe complexity and allowed for reproducibility of hybridization signals. PCR amplification eliminated the need for subsequent probe preparations by alkaline lysis. As a guideline, we used 3-4 µl "PCR template" for any 100 µl PCR labeling reaction. If two probes were labeled in the same vial, we used 3 µl of each "PCR template". 4-5 µl labeled PCR product per probe was used for one hybridization [for example, for mouse chromosome 1 (Table 1), probes 1c and 1t were labeled in the same vials. 8 µl each of R6G-, DNP- and BIO-labeled PCR products of 1c+1t were used for one hybridization].

Antibody detection, imaging and image analysis.

All antibodies used for detection were stored as 1mg/ml stock solutions and were used at 1:100 dilutions, in 100 µl 4xSSC/slide. All antibody incubations were done for 10 minutes at 37° C and were followed by 10-15 minute washes in 4xSSC/0.1% Tween20, at 37 to 42° C. Some antibodies were purchased labeled, others were custom labeled in our laboratory, using standard protocols (Molecular Probes, Amersham-Pharmacia Biotech). When only BIO, DIG and DNP were used to label probes, the detection required only one layer of antibody (one step detection), including mouse antiDIG-FITC (Sigma), avidin-Cy5 and rat antiDNP (Accurate Chemical) custom labeled with Cy3.5 (Pharmacia-Amersham). For the more complex 1-5 scheme, several tests were done with antibodies from several manufacturers, to find a combination of proteins which did not cross-react non-specifically with one another. These experiments resulted in the following detection/amplification scheme: FITC was detected with goat antiFITC followed by donkey antigoat-DTAF (Accurate Chemical). Rhodamine was detected with rabbit antirhodamine (Molecular Probes) custom-conjugated with Cy3 (Pharmacia-Amersham) followed by donkey antirabbit (Accurate Chemical) custom-conjugated with Cy3. BIO was detected with one layer of Avidin-Cy5. DNP was detected with rat antiDNP-Cy3.5. DIG was detected with sheep antiDIG (Boehringer Mannheim) custom conjugated with DEAC (Molecular Probes) followed by donkey antisheep (Accurate Chemical) custom conjugated with DEAC. All antibodies were combined into three detection steps/vials: in the first step, we mixed in the same vial goat antiFITC, rabbit antirhodamine-Cy3, sheep antiDIG-DEAC and rat antiDNP-Cy3.5. As our available goat antiFITC was binding non-specifically biotin, we added 1µl of a 1mM custom-made BIO-dUTP to the antibody mix immediately before using the antibodies. After 10 minutes antibody incubation and 10-15 minutes post-antibody wash, the slide was subjected to the second step of detection: this included donkey antigoat-DTAF and donkey antirabbit-Cy3. After the usual 10 minute incubation and 10-15 minute wash, a third layer of detection was added, which included avidin-Cy5 and donkey antisheep DEAC. To block the weak non-specific binding of our

donkey antisheep to the goat antiFITC antibody used at the first layer, 1 μl of a 1mg/ml goat IgG solution was added in the same vial with avidin and donkey antisheep, prior to using them. After incubation and washing, the slide was stained with DAPI, mounted with antifade solution, and examined at the microscope using appropriate filters (Henegariu et al, 2000). Gray-scale images of every channel were captured on an Olympus Provis microscope, equipped with a Photometrix Sensys camera, using the M-FISH software (PSI Inc). Gray scale images were pseudocolored in groups of three in Adobe Photoshop (creating RGB images). In the first combination (= FR), FITC was pseudocolored green, rhodamine/Cy3 red and DAPI blue. In the other combination (= NBD), DNP-Cy3.5 was pseudocolored red, BIO-Cy5 blue and DIG-DEAC green.

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Figure legends.

Fig. 1a-c. TM-FISH analysis of a case with 46,XX add(1)(1q41), based on the *1/5 detection scheme*. Using Photoshop (Adobe), grayscale images of the same metaphase in FITC (F), rhodamine-Cy3 (R), DNP-Cy3.5 (N), BIO-Cy5 (B), DIG-DEAC (D) and DAPI channels were combined and pseudocolored into two RGB images : FR (including FITC-green, Rhodamine-red and DAPI-blue) and NBD (including DNP/Cy3.5-red, BIO/Cy5-blue and DIG/DEAC-green). The arrows in ***a*** and ***b*** indicate the extra material on 1qter with a fluorescent signal carrying the color signature of chromosome 8 telomeres. Numbers in ***a*** show the position of all human chromosomes. The two small arrows in the upper right corner point to the same translocation as detected by M-FISH (very weak signal) or G-banding (the q arm of human chromosome 1). For a better signal visualization, in ***c***, the image of one chromosome of every human pair was split in its original grayscale images. The vertical lines separate the FR from NBD images. The first chromosome in every group as well as the first chromosome after the vertical white line depicts the merged color image, as it appears in figures ***a*** and ***b***. The white circle indicates the DAPI image of that chromosome. The red, green or blue dots above each chromosome indicate the pseudo-color assigned for that channel in the respective RGB image (FR or NBD). The same legend appears in the bottom right corner of ***c***. The color chart in ***d*** depicts the expected color of every human subtelomeric probe in the FR and NBD images respectively, and also indicates (letters) what haptenes (see discussion above) were used to label each telomere. For example, chromosome 1 telomeric probes were colored red in the FR image and magenta in NBD, indicating that they were labeled by rhodamine (R), DNP (N) and BIO (B). The *2/3 detection scheme* (not shown) can be displayed the same way as in ***a*** and ***b***, the only difference being that the two images, FR and NBD, come from different metaphases. The same chart (**d**) can be used for the mouse TM-FISH, with some exceptions, also shown in Table 1. Thus, mouse chromosome 5 was labeled FNB (green in the FR and magenta in the NBD images - similar to human 21); chromosome 6 was labeled R (red in the FR image - similar to human 5) and X was labeled N (red in the NBD image - similar to human 20). No probes for the mouse Y chromosome were used.

Fig. 2. *a-c*. The human 3/3 detection scheme, using all telomere probes combined in three sets (#1 in ***a***, #2 in ***b*** and #3 in ***c***). Probes were labeled with BIO, DIG and DNP, thus there were only three initial colors (red, green and blue, identical to the NBD images in Fig 1). The colored dots indicate the expected color of every chromosome pair in each of the three sets and provide the labeling scheme for every chromosome. As in figure 1, red

is N, green is D, blue is B, cyan is BD, magenta is NB, yellow is BD and white is NBD. Note that in the 3/3 detection scheme, each probe set detects the chromosomes independently from the other two sets. For chromosomes 20 in set #1 (***a***) and 19 in set #2 (***b***), we used ratio labeling for detection. The telomeric probes for these two chromosomes yield signals significantly smaller than the signals for chromosomes 4 (***a***) and 7 (***b***), respectively. This obvious signal difference, allows chromosome identification. ***d-f.*** Mouse TM-FISH images (1/5 strategy): as all mouse chromosomes are acrocentric, only one probe is close to the telomere, the other one is close to the centromere. Images were prepared as described for the human probes in Fig 1, with FR (in ***d***) and NBD (in ***e***). Arrows point toward the two chromosomes carrying the reciprocal t(4;9) translocation. The reverse DAPI image of the same metaphase is also shown (e). The white letters indicate the positions of the derivative chromosomes 4 (A) and 9 (B) in the metaphase.

Table 1. Mouse and human probe combinations for TM-FISH analysis

Chr	Clone name	Mouse TM-FISH					Chr	Clone name	Human TM-FISH 1/5 and 2/3 combinations					Chr	Human TM-FISH 3/3 combinations			
		F	R	N	B	D			F	R	N	B	D		D	N	B	
1c	45C1		1	1	1		1p	13p11		1	1	1		1p	1	1	1	
1t	39L15						1q	160h23						1q				
2c	49N22	2	2	2			2p	68j13	2	2	2			4p			4	
2t	20L10						2q	210e4						4q				
3c	33D15			3	3	3	3p	*B47a2*			3	3	3	9p	9		9	
3t	443K17						3q	196f4						9q				
4c	39B18	4				4	4p	36p21	4				4	10p	10			#1
4t	362D3						4q	*cT55*						10q				
5c	48H24	5		5	5		5p	114j18		5				13q		13	13	
5t	40J4						5q	240g13						17p		17		
6c	45K20		6				6p	36i2		6		6	6	17q				
6t	51F24						6q	57h24						20p			20	
7c	43A19				7	7	7p	164d18				7	7	20q				
7t	100O21						7q	3k23						22q	22	22		
8c	20H8	8		8			8p	63m14	8		8			2p		2	2	
8t	38K2						8q	*2053b3*						2q				
9c	23D13			9		9	9p	34h2			9		9	5p	5			
9t	55J6						9q	112n13						5q				
10c	47K7		10			10	10p	305f4		10			10	7p			7	
10t	26C12						10q	*2136a1*						7q				
11c	39E20	11	11				11p	*2209a2*	11	11				8p		8		
11t	434L24						11q	*2072c1*						8q				#2
12c	47N14	12			12		12p	90i5	12			12		11p	11	11		
12t	34I19						12q	221k18						11q				
13c	50K9			13	13		13q	85a10			13	13		15q	15	15	15	
13t	38I3						14q	*2006a1*	14					16p	16		16	
14c	429N17	14					15q	154p1		15		15		16q				
14t	48O2						16p	119l16	16		16		16	19p			19	
15c	43G16		15		15		16q	240g10						19q				
15t	62I2						17p	*2111b1*		17	17			3p			3	
16c	50I1	16		16		16	17q	362k4						3q				
16t	43D12						18p	52m11				18		6p	6		6	
17c	65C22		17	17			18q	*2050a6*						6q				
17t	50F18						19p	*F20643*					19	12p	12	12		
18c	53M14				18		19q	*F21283*						12q				#3
18t	51B23						20p	*2005a4*			20			14q		14	14	
19c	26B5					19	20q	204a16						18p	18			
19t	49P14						21q	63h24	21		21	21		18q				
Xc	51A6			X			22q	99k24		22	22		22	21q	21	21	21	
Xt	23H12						XYp	98c4	xy			xy	xy	XYp		xy		
							XYq	*c8.1/2*						XYq				

"Chr" = chromosome number; "c", "t" for mouse clones = the centromeric or telomeric end of the chromosome. "p", "q" for human clones = indicate the p and q arm of the chromosome. F = FITC; R = R6G; N = DNP; B = BIO; D = DIG. Numbers within the table indicate the fluors or haptenes with which the probes for the respective chromosome were labeled. We used numbers, so that chromosome identification is easier. Italic characters of some human clone names indicate the cosmid probes. All other human probes were P1, PACs. When human probes are used according to the 2/3 approach, FITC is replaced by BIO, and R6G by DIG. The probes in these two columns are then hybridized on one area of the slide, whereas the rest of the human clones (the ones always labeled by BIO, DIG, DNP) are hybridized on another area of the slide. Note: to differentiate the chromosomes, information from *both* hybridizations needs to be combined. #1-#3 = the three probe mixes used for the 3/3 TM-FISH combination. Probes corresponding to chromosomes 4 and 20 (mix #1) and chromosomes 7 and 19 (mix #2), respectively, are differentiated by the size of the fluorescent signal, not by combinatorial labeling.

Fig.1

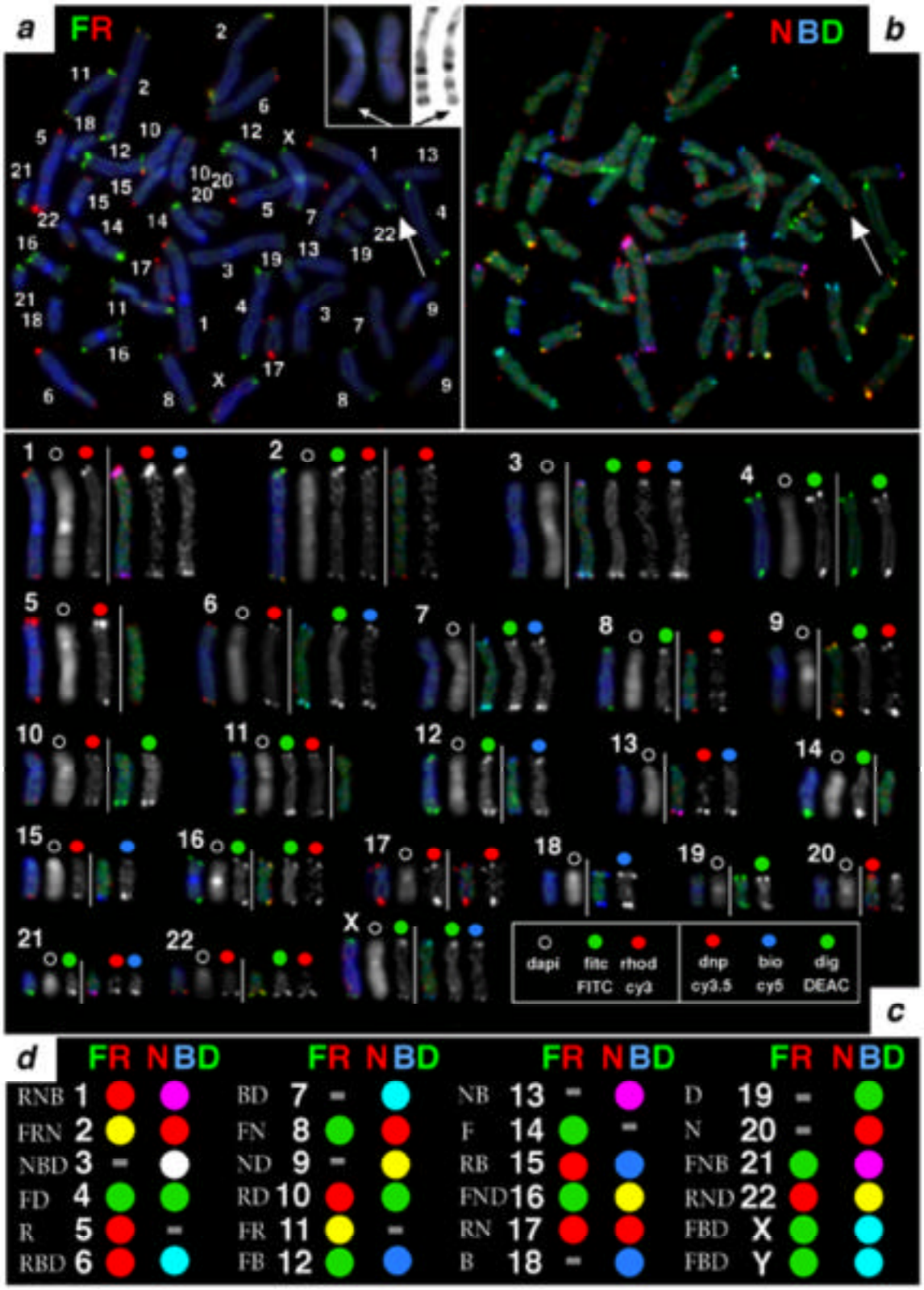
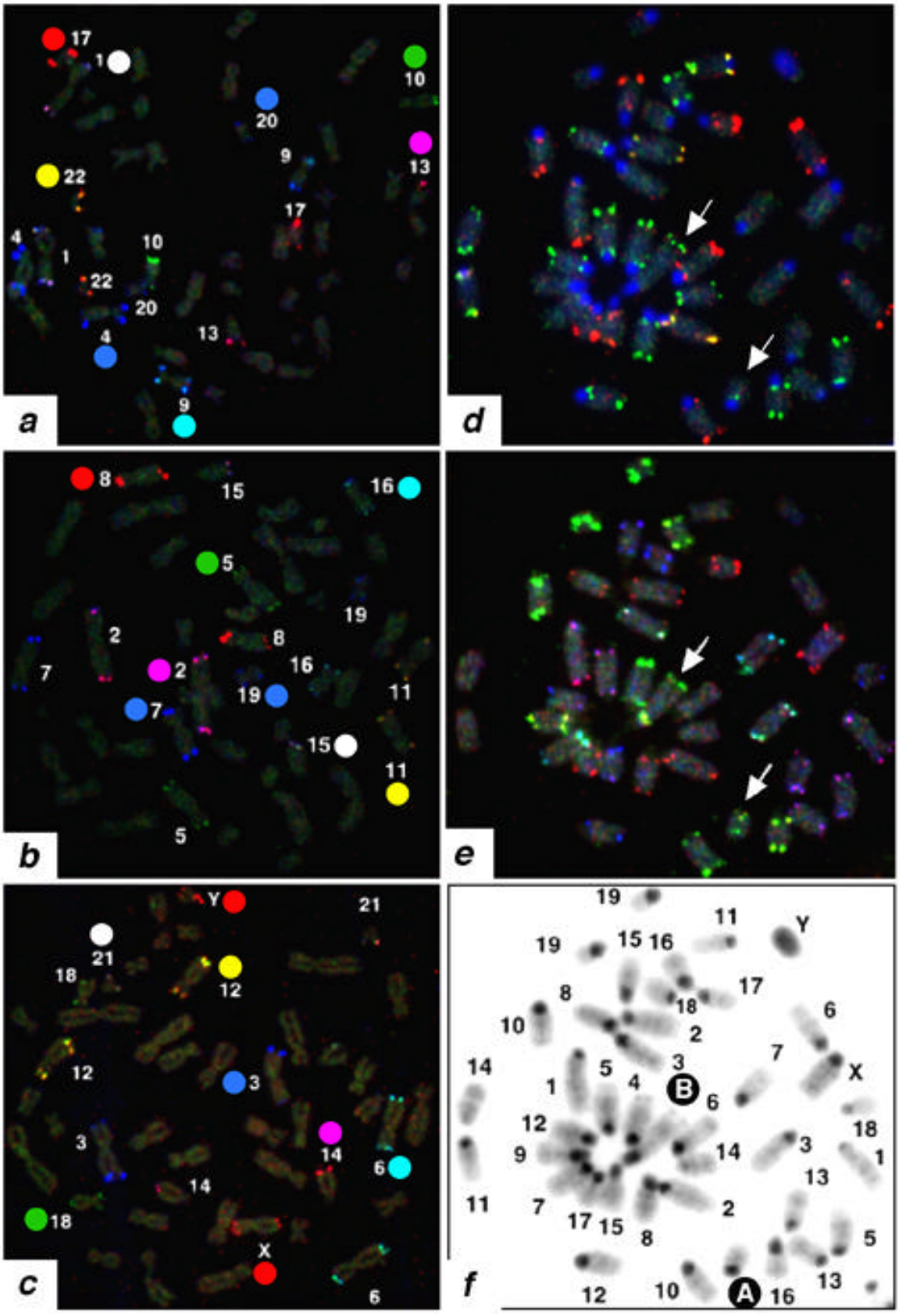

a
FR
b
NBD
c
dapi
fitc
FITC
rhod
cy3
dnp
cy3.5
bio
cy5
dig
DEAC
d
FR NBD
RNB 1
FRN 2
NBD 3
FD 4
R 5
RBD 6
BD 7
FN 8
ND 9
RD 10
FR 11
FB 12
NB 13
F 14
RB 15
FND 16
RN 17
B 18
D 19
N 20
FNB 21
RND 22
FBD X
FBD Y

Fig.2


a
17
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22
17
4
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22
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13
9
d
b
8
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16
5
19
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8
7
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16
19
11
7
15
11
5
e
c
Y
21
21
18
12
12
3
3
14
6
14
18
X
6
f
19
11
Y
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14
5
3
X
1
4
B
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12
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A
16
13